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Hydrophobic Interactions of *n*-Alkyl Diamines with the *N*-Methyl-D-aspartate Receptor: Voltage-Dependent and -Independent Blocking Sites

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SUMMARY

We examined the block of *N*-methyl-D-aspartate (NMDA) receptors by *n*-alkyl (straight chain) diamines and related monoamines and triamines using whole-cell voltage clamp recording of NMDA receptor currents in cultured rat hippocampal neurons and [^{3}H] dizocilpine binding to rat forebrain homogenates. At −60 mV, the diamines (carbon chain lengths 3–12) produced a concentration-dependent inhibition of NMDA receptor current (IC_{50} values, 6128–7.3 μM). For diamines of carbon chain lengths greater than 6, the inhibition was partially, but not completely, relieved by depolarization, indicating that the block occurs at distinct voltage-dependent and voltage-independent sites. The block produced by short-chain diamines (carbon chain lengths 3–6) was completely relieved by depolarization, indicating little or no interaction with the voltage-independent site. In comparison with the corresponding diamines, homologous monoamines exhibited very low potency, whereas homologous triamines were of equal or lower potency. For long-chain diamines, inhibitory potency at both the voltage-dependent and voltage-independent sites was correlated with carbon chain length (binding energy increasing 600–700 cal/mol-CH_2), suggesting that binding to each of the sites is stabilized by a hydrophobic interaction. Affinities for the voltage-dependent blocking site (transformed to 0 mV) and for the voltage-independent blocking site were similar. These values were also similar to the inhibitory potencies of the diamines in the [^{3}H]dizocilpine binding assay. Analysis of the voltage-dependence of block at the voltage-dependent site yielded $z\delta$ values for diamines of intermediate length (carbon chain lengths 7–9) that decreased with increasing length from 0.91 to 0.63 [approaching the $z\delta$ values of monovalent blockers (~0.54) and one-half of the $z\delta$ values of shorter diamines (~1.1)], suggesting that the intermediate length diamines block in a linear, extended chain conformation with one of the charges having incomplete access to a deep binding site. Longer chain diamines (carbon chain lengths 10 and 12) exhibited larger $z\delta$ values (0.78 and 0.98, respectively), presumably because enhanced conformational flexibility permitted a folded-over conformation. From the intercharge distances of the intermediate length diamines in their lowest energy conformation, we estimated that the total voltage drop within the NMDA receptor channel occurs over a distance of ~20 Å. The putative polyamine facilitatory site antagonist diethylenetriamine inhibited NMDA-induced currents at the voltage-dependent site (IC_{50}, 654 μM; −60 mV). However, at positive potentials, diethylenetriamine neither produced block by itself nor reversed the inhibitory effect of diamines, indicating that it is not an antagonist at the voltage-independent blocking site. We conclude that the NMDA receptor-complex possesses two distinct hydrophobic diamine blocking sites, one of which is voltage-dependent and the other which is not. The available evidence suggests that these sites are distinct from the site at which polyamines such as spermine allosterically facilitate channel opening.

The NMDA receptor is a member of the ionotropic glutamate receptor family of ligand-gated ion channels that mediate excitatory neurotransmission in the central nervous system. Glutamate and glycine are coagonists, both of which must be present for gating of the NMDA receptor channel, whereas a wide variety of other agents, including the polyamines spermine and spermidine, allosterically modulate NMDA receptor function (1). The action of polyamines on the NMDA receptor was initially characterized with radioligand binding using the uncompetitive (open channel) ligand [^{3}H]dizocilpine (2, 3). The tetramine spermine and the triamine spermidine enhanced [^{3}H] dizocilpine binding at low concentrations and inhibited binding at higher concentrations, giving rise to bell-shaped concentration-response curves (2, 3). In contrast to spermine and spermidine, the diamine 1,10-diaminodecane (DA10) (4) and other long-chain *n*-alkyl diamines (5) produced only concentration-

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MA*x* and DA*x*, *n*-alkyl monoamine and diamine, respectively, of carbon chain length *x*; DET, diethylenetriamine; BHT, *bis*-hexamethylenetriamine; EPPS, *N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulfonic acid]; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol *bis*(β-aminoethyl-ether)-*N*, *N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-[2-ethanesulfonic acid].

dependent inhibition of [^{3}H]dizocilpine binding. These *n*-alkyl diamines have also been observed to cause a shift in the spermine concentration-response curve, suggesting that they are competitive antagonists at the site where polyamines act to facilitate [^{3}H]dizocilpine binding. Similar evidence has been reported for the triamine diethylenetriamine (DET) (4). DET has also been shown to decrease the inhibitory effect of DA10, leading to the proposal that the latter compound acts as an inverse agonist at the polyamine facilitatory site (4).

Recent electrophysiological studies indicate that compounds classified either as polyamine antagonists or inverse agonists on the basis of radioligand binding experiments may inhibit NMDA receptors through an open channel blocking mechanism. For example, we have shown that the putative polyamine antagonist arcaine causes a voltage-dependent, open channel block of NMDA receptor responses in cultured hippocampal neurons (6). Subsequently, the *n*-alkyl diamines DA10 and 1,12-diaminododecane (DA12) were also observed to inhibit NMDA receptor responses (7, 8). A proportion of the diamine block, like that of arcaine, is voltage-dependent, and single-channel recording studies support the view that the voltage-dependent block occurs by an open channel mechanism. Recently, Benveniste and Mayer (9) have confirmed that arcaine and DA10 produce a voltage-dependent block of NMDA receptor current. Additionally, these investigators observed a similar inhibitory effect of DET and, in contrast to the results of the binding studies, demonstrated that the facilitatory actions of polyamines are not antagonized by arcaine, DA10, or DET.

In the present study, we sought to characterize in more detail the structural requirements for block of NMDA receptors by diamines of various chain lengths. Our results indicate that the inhibitory actions of diamines are mediated through two distinct sites, one situated deep (50–60%) within the transmembrane electric field and another located superficial to the electric field. The characteristics and structure-activity relationships for block at each site allow inferences about certain structural features of the NMDA receptor-channel complex.

Experimental Procedures

Cell culture. Rat hippocampal neurons were grown in primary culture as previously described (10). In brief, hippocampi from 19-day-old Sprague-Dawley rat embryos were triturated in modified minimal essential medium with Earle's salt (Advanced Biotechnologies, Columbia, MD) by repeated passage through a 10-ml pipette. The cell suspension was plated at a density corresponding to 1–1.5 hippocampi/dish on 35-mm polystyrene Petri dishes (Falcon 3001; Becton Dickinson Labware, Oxnard, CA) that were precoated with Matrigel (Collaborative Biomedical Products, Bedford, MA). The plating media was supplemented with 10% horse serum (GIBCO, Grand Island, NY), 10% fetal calf serum, N3 (20 mg/ml transferrin, 200 μM putrescine, 60 nM sodium selenite, 20 ng/ml triiodothyronine, 10 mg/ml insulin, 40 nM progesterone, 40 ng/ml corticosterone) (11, 12), and 1% glutamine. The cultures were maintained at 37°C in a humidified atmosphere containing 10% CO_2. After 6 days, a few drops of fresh media (without fetal calf serum and N3) were added to the cultures. The cells were used for recording within the next 6–13 days.

Whole-cell recording. Electrophysiological recordings were carried out at room temperature (25°C) on the stage of an inverted phase-contrast microscope. Before each experiment, the culture medium was removed, the cells were rinsed completely, and the culture dish was partially filled with bathing medium containing (in mM) 140 NaCl, 5 KCl, 0.1 $CaCl_2$, and 10 HEPES. The bathing medium also contained 1 μM tetrodotoxin to block voltage-activated Na^+ channels and 1 μM strychnine to block glycine-activated Cl^- channels. The osmolality was adjusted to 315–325 mosM with sucrose and to a pH of 7.4 with NaOH.

Patch pipettes (3–6 MΩ) were prepared from filament-containing thin wall glass capillary tubes (1.5 mm outside diameter, World Precision Instruments, New Haven, CT) using a two-stage vertical pipette puller (Model L/M-3P-A, List Medical, Darmstadt, Germany). The electrodes were filled with recording solution containing (in mM) 145 CsCl, 2 $MgCl_2$, 5 HEPES, 0.1 $CaCl_2$, and 1 EGTA. The osmolality was adjusted to 310 mosM with sucrose and to a pH of 7.4 with CsOH.

Whole-cell recordings were performed with an Axopatch 1C or 200A patch-clamp amplifier (Axon Instruments, Burlingame, CA) and displayed on a high-speed ink pen recorder (Gould Electronics, Cleveland, OH) and digitized for storage on optical media. Unless otherwise noted, the holding potential was maintained at −60 mV.

Drug perfusion. Drug solutions were applied via a rapid perfusion system consisting of a seven-barrel perfusion device in which all barrels emptied via a common tip (~600 μm inside diameter) (13). The tip of the perfusion device was positioned ~800 μm from the cell under study. The flow through each barrel was gravity fed and regulated by microvalves operated by a programmable microprocessor-based controller. One barrel was used to apply a wash solution alone, whereas the others applied perfusion solution containing various drugs alone and in combination. Only one valve was opened at a time. Switching between solutions occurred within <10 msec (14). Drugs were dissolved on the day of use in perfusion solution consisting of recording solution containing 10 μM glycine to saturate the glycine site on NMDA receptors. Drug solutions were adjusted to pH 7.4.

[^{3}H]Dizocilpine binding assay. [^{3}H]Dizocilpine binding assays were carried out as described previously (15). Briefly, freshly removed rat forebrains were homogenized in 0.32 M sucrose and 5 mM EDTA (pH 7.0) using a Polytron (Kinematica, Switzerland). The homogenate was centrifuged at 1000 ×*g* for 10 min and the supernatant was collected and centrifuged at 36,000 ×*g* for 30 min. The pellet was then resuspended in 5 mM EDTA (pH 7.45) and centrifuged at 50,000 ×*g* for 30 min. This step was repeated twice before reconstituting the membranes in 30 mM EPPS and 1 mM EDTA (pH 7.45) and repeating the centrifugation cycles three more times. After the final cycle, the homogenate was reconstituted in a final volume of 5 ml/brain. Incubations were carried out at room temperature for 2.5 hr in the presence of 4–6 nM [^{3}H]dizocilpine, 100 μM glutamate, and 100 μM glycine. Nonspecific binding was determined in the presence of 200 μM ketamine. The final volume of the incubation mixture including drug additions was 0.5 ml, and the final protein concentration was between 100 and 200 μg/tube. Incubations were terminated by filtration using a Brandel cell harvester and the filters were washed with 9 ml of ice-cold buffer. The filters were soaked in a liquid scintillation mixture overnight and counted in a liquid scintillation counter at an efficiency of 57%.

Data analysis. The fractional block (B) of NMDA-evoked currents in the whole-cell recording experiments was calculated according to the formula $B = 1 - I_B/I$, where I is the steady-state current evoked by NMDA and I_B is current evoked by NMDA in the presence of the blocker. Concentration-effect data were fitted to the logistic equation

$$B = 1/\{1 + (IC_{50}/[D])^{n_H}\} \quad (1)$$

where [D] is the blocker concentration, IC_{50} is the concentration resulting in 50% block, and n_H is an empirical parameter describing the steepness of fit and having the same meaning as the Hill coefficient. All quantitative data are expressed as mean ± SE; n is the number of neurons tested.

Drugs. NMDA and ketamine were obtained from Research Biochemicals (Natick, MA). [^{3}H]Dizocilpine (25.7 Ci/mmol) was obtained from New England Nuclear-Dupont (Wilmington, DE). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Aldrich (Milwaukee, WI).

Results

Diamine block of NMDA-induced currents at −60 mV. A series of *n*-alkyl diamines (chain lengths 3–12) were examined for their effects on whole-cell currents induced by 10 μM NMDA (+ 10 μM glycine). At a holding potential of −60 mV, all of the diamines caused a concentration-dependent block of NMDA-induced currents (Figs. 1 and 2). As illustrated in Fig. 1, onset and recovery from block occurred rapidly (<100 msec). The IC_{50} values derived from the concentration-response curves shown in Fig. 2 are given in Table 1. It is apparent that for diamines of carbon chain lengths greater than 6, blocking potency at −60 mV is positively correlated with chain length. In order to further define the structural requirements for the blocking effect, we examined the monovalent and trivalent analogs of DA12, *n*-dodecylamine (MA12; single terminal amine), and bishexamethylenetriamine (BHT; two terminal amines and a secondary amine bisecting the carbon chain). MA12 tended to cause loss of the voltage-clamp [presumably due to the detergent properties of the molecule (16)] and was therefore difficult to evaluate; however, concentrations as high as 100 μM caused only a 22 ± 4% (n = 4) inhibition of the current, so that it was substantially weaker than DA12. BHT had higher potency than MA12, but was still 30-fold weaker than DA12 (Table 1). A similar comparison was made between DA4, the analogous four-carbon monoamine *n*-butylamine (MA4), and the four-carbon triamine DET. MA4 was a weak blocker, producing no block at 100 μM and only 14.9 ± 1.4% block at 300 μM (n = 5). DET was substantially more potent than MA4, and was roughly equivalent to DA4 (Table 1). These results indicate that two positive charges are required for optimal blocking potency and that additional charges may or may not reduce potency.

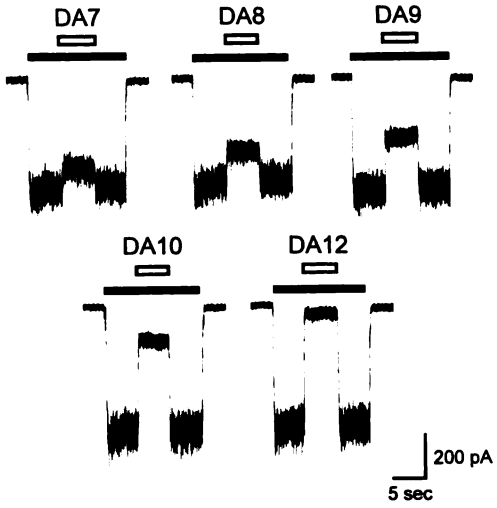

Fig. 1. Inhibition of NMDA-induced inward currents by diamines of various chain lengths. *Filled bars* indicate periods of 10 μM NMDA (+ 10 μM glycine) application. *Open bars* indicate periods of DA7, DA8, DA9, DA10, and DA12 coapplication. Diamines were applied at a concentration of 100 μM. All records are from the same cell. Holding potential, −60 mV.

Voltage-dependence of the diamine block. The voltage-dependence of the block of NMDA receptor current by the diamines was examined by determining the fractional block at holding potentials of −100 to +60 mV. Sample records illustrating the voltage-dependence of the block produced by 3 mM DA5, 3 mM DA7, and 30 μM DA12 are shown in Fig. 3, *left*. For each of the three diamines, the fractional block was greater at −60 mV than at +60 mV. However, there was a marked difference among the diamines in the extent to which the block was relieved at the positive holding potential. Thus, at the concentrations tested, all three diamines produced a comparable (nearly complete) block of the NMDA current at −60 mV. However, at +60 mV, the block produced by DA5 was nearly completely relieved, whereas for DA7 and DA12, a substantial component persisted at the depolarized potential. The mean relative current values at various holding potentials are plotted in Fig. 3, *right*. There was a sigmoidal relationship between holding potential and relative current that plateaued at a value close to 1 for DA5 and at values substantially less than 1 for DA7 and DA12. The component of the block produced by DA7 and DA12 that was not relieved by depolarization presumably occurs at a site distinct from that of the voltage-dependent inhibitory effect (*i.e.*, at a voltage-independent

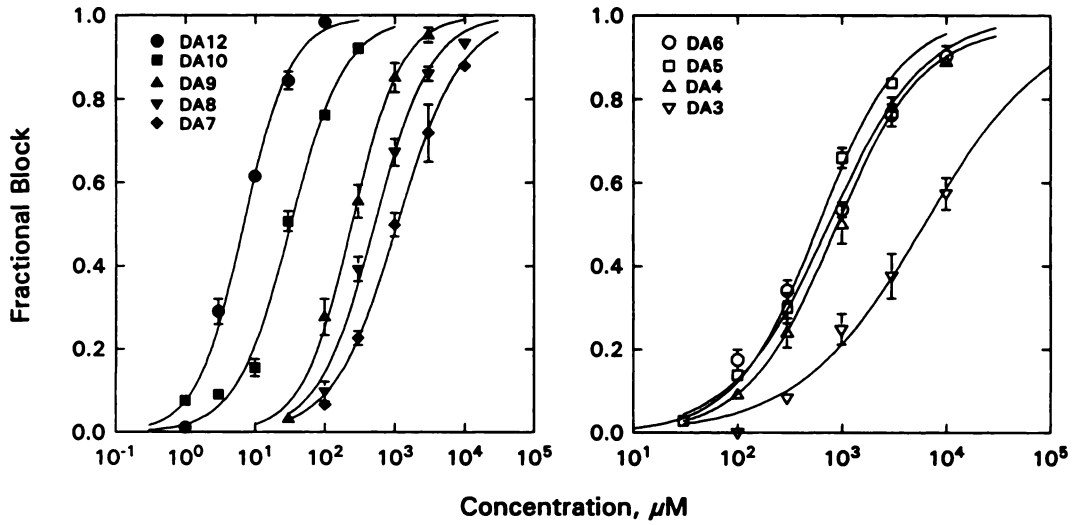

Fig. 2. Concentration-response data obtained from experiments similar to those shown in Fig. 1. Each point represents the mean ± SE of data from 4–7 neurons. The curves show the best fits to the data calculated according to Eq. 1. The IC_{50} values obtained from the fits are given in Table I; the n_H values were 0.97–1.3.

TABLE 1

Inhibitory potencies of polyamines against NMDA-induced current and [^{3}H]dizocilpine binding

$NH_2(CH_2)_nNH_2$: n =	NMDA current (−60 mV)	IC_{50}: NMDA current (+40 mV)	[^{3}H]Dizocilpine binding
		μM	
3	6128	Inactive	>10 mM
4	859	Inactive	>10 mM
5	536	Inactive	>10 mM
6	918	>10 mM	>10 mM
7	981	5108	5380
8	513	1707	883
9	244	558	218
10	33	135	41
12	7.3	32	24
BHT[a]	235	570	ND[b]
DET[c]	654	Inactive	ND

[a] Bishexamethylenetriamine [$NH_2(CH_2)_6NH(CH_2)_6NH_2$].
[b] Diethylenetriamine [$NH_2(CH_2)_2NH(CH_2)_2NH_2$].
[c] ND, not determined.

blocking site). After Woodhull (17), the voltage-dependent affinity for binding of a polyamine to its voltage-dependent blocking site can be expressed as

$$K_D(V) = K_D(0) \exp(z\delta FV/RT) \quad (2)$$

where $K_D(0)$ is the dissociation constant of the polyamine-binding site complex at a transmembrane potential of 0 mV, δ is the fraction of the total electric field sensed at the binding site, z is the charge of the polyamine and F, R and T have their usual meanings. Assuming that the channels are nonconducting when occupied by a single polyamine molecule, the relative current in the presence of polyamine at concentration [D] can be expressed by incorporating Eq. 2 in a binding isotherm to obtain

$$I/I_o = (1 - \beta)[1 + [D]/K_D(0) \exp(-z\delta FV/RT)]^{-1} \quad (3)$$

where I and I_o are the currents in the presence and absence of the polyamine, respectively, and β is the fraction of the block that is voltage-independent. The smooth curves in Fig. 3, *right* demonstrate the best fits to the mean fractional current values according to Eq. 3. The β values for DA5, DA7, and DA12 are 0, 0.38, and 0.58, repectively, indicating increasing affinity of the diamines for the voltage-independent blocking site with increasing chain length. Affinities of the complete series of aliphatic amines for the voltage-independent blocking site were estimated by determining the IC_{50} values from concentration-block curves at a holding potential of +40 mV (Fig. 4). Diamines of carbon chain lengths less than 7 were inactive at this potential, as was DET; however, the longer chain diamines and BHT were capable of producing complete block. The IC_{50} values of the active diamines are presented in Table 1 and are also plotted in Fig. 5. The relative apparent free energy values for binding of the diamines were estimated according to the relationship $\Delta G = RT \ln IC_{50}$ (where the IC_{50} is compared to an arbitrary standard-state concentration of 1 M). The best straight line fit to these data has a slope of 670 cal/mole-CH_2, which corresponds favorably with the energy required to transfer one methylene group in a model n-alkane from a nonpolar (hydrocarbon) to an aqueous environment (18) and also for binding of n-alkyl substituted quaternary ammonium compounds to a hydrophobic acceptor site on K^+ channels (19, 20). These results suggest that binding of the diamines to the voltage-independent blocking site is stabilized by the interaction of the alkane chain with a hydrophobic site on the channel.

We next investigated the affinities of the diamines and certain homologous monoamines and triamines for the voltage-dependent blocking site. $K_D(0)$ values were estimated from the data presented in Fig. 2 by least squares fitting to a linearized form of Eq. 3 (see caption to Fig. 5); estimates of the $z\delta$ values were derived from the slopes of these fits (Fig. 6A). The $K_D(0)$ values obtained in this way are plotted in Fig. 5. It is apparent that for diamines of chain lengths greater than 5, affinity increases exponentially with increasing chain length. The binding energy per methylene obtained from a fit to the data (number of CH_2 groups > 5) was 610 cal/mole-CH_2, suggesting, as in the case of the voltage-independent site, that binding is stabilized by an hydrophobic interaction.

For diamine binding to the voltage-dependent blocking site, the steepness of the exponential relationship between apparent affinity [$K_D(V)$] and voltage is measured by the quantity $z\delta$. The $z\delta$ values of the diamines and several monoamines and triamines are presented in Fig. 6B. From this figure, it is apparent that diamines of short (DA4, DA6) and long (DA12) chain length have $z\delta$ values in the range 1–1.2, whereas intermediate chain length diamines have lower $z\delta$ values that approach the value of the monoamines and triamines, which is roughly one-half the maximum $z\delta$ value. The distinction between the intermediate chain length diamine and short-chain diamines, monoamines, and triamines is illustrated in Fig. 6A, which compares the slopes of the best fit lines for representative compounds of each of these classes. Fits to the data for DA9, a

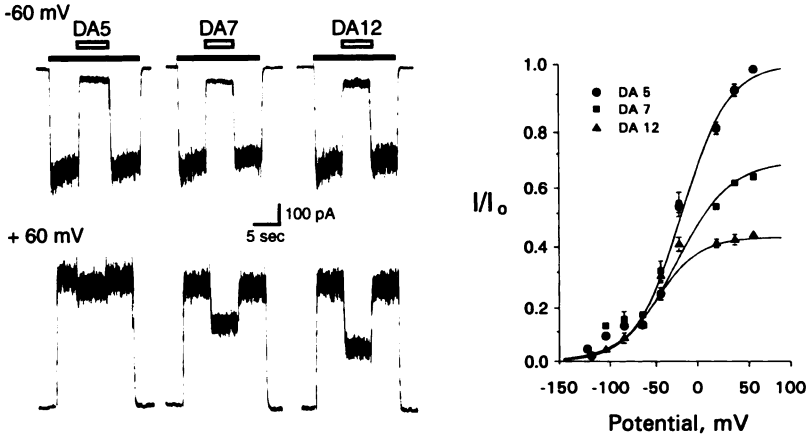

Fig. 3. Voltage-dependent block of NMDA-induced current by diamines. *A*, current traces showing the block by 3 mM DA5, 3 mM DA7 and 30 μM DA12 at −60 and +60 mV. *B*, relative current values for the diamines at different holding potentials. Each point represents mean ± SE from 4–6 cells. The smooth curves indicate the best fits to the data points according to text Eq. 3. The fitted parameters are given in the text and in Table I.

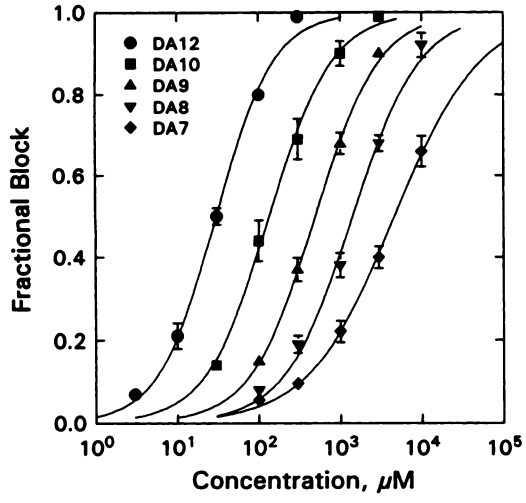

Fig. 4. Concentration-response data for block of NMDA-induced current by diamines at +40 mV. Each point represents the mean ± SE of data from 4–8 cells. The curves show the best fits to the data calculated according to Eq. 1. The IC_{50} values obtained from the fits are plotted in Fig. 5; the n_H values were 0.9–1.3.

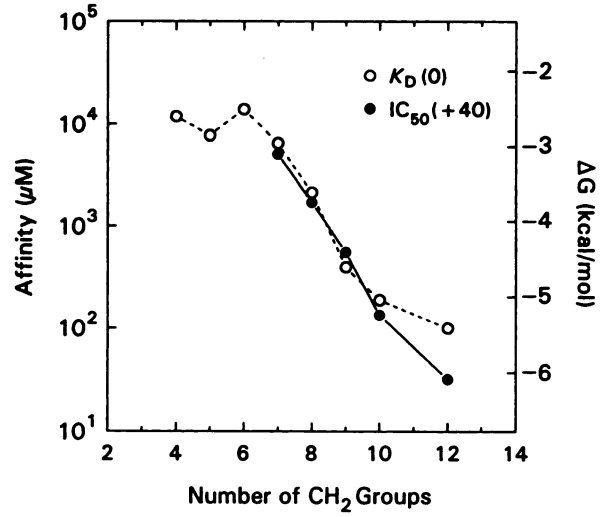

Fig. 5. Correlation between carbon-chain length of the diamines and their potencies in blocking NMDA-induced currents via interaction with the voltage-dependent [$K_D(0)$] and voltage-independent [$IC_{50}(+40)$] blocking sites. $K_D(0)$ values were determined from linear least square fits to transformations of the data presented in Fig. 2 according to a linearized form of text Eq. 3: $\ln [I_o/I(1-\beta) - 1] = \ln \{[D]/K_D(0)\} - z\delta FV/RT$. The $z\delta$ values are presented in Fig. 6B.

representative short-chain diamine, MA6, a monoamine, and DET, a triamine, have similar slopes, whereas the slope of the line fitted to the DA6 data is substantially steeper. Pairwise comparisons using the test for parallelism indicated that the slopes of MA6, DA9, and DET fits were not significantly different, whereas the slope of the DA6 fit was significantly different from the MA6 and DET fits with $p < 0.01$ and from DA9 with $p < 0.05$.

Failure of DET to interact with the voltage-independent blocking site. Based on radioligand binding studies, it has been proposed that DET is an antagonist at the site where polyamines act to facilitate NMDA receptor function (4). However, as noted above, in our electrophysiological experiments, DET produced a voltage-dependent block of NMDA-induced currents similar to that of the diamines. Even at DET concentrations that were 5-fold greater than the IC_{50} at −60 mV, the blocking action of DET could be completely reversed at +40 mV (Table 1), indicating that DET does not produce voltage-independent block. To investigate whether DET can antagonize the inhibition produced by long-chain diamines acting at the voltage-independent blocking site, we examined the effects of a high concentration (3 mM) of DET on the block produced by 100 μM DA10 at +40 mV. In 6 cells, DA10 produced a 60.0 ± 2.7% block of the NMDA-induced current. In the presence of 3 mM DET, DA10 still produced 59.9 ± 4.7% block. These results indicate that DET does not act as an antagonist at the voltage-independent blocking site.

Inhibition of [^{3}H]dizocilpine binding. The diamines were evaluated for their ability to inhibit [^{3}H]dizocilpine binding to NMDA receptors in rat forebrain membranes. Diamines of chain lengths 3–6 caused less than 50% inhibition of binding at concentrations up to 10 mM (Table 1). The longer diamines were more potent, producing concentration-dependent inhibition of binding, as shown in Fig. 7. The IC_{50} values (summarized in Table 1) indicate a monotonic increase in equilibrium binding affinity with increasing chain length, as was the case for the binding affinities to the voltage-dependent [$K_D(0)$] and voltage-independent [$IC_{50}(+40)$] sites obtained in the whole-cell voltage clamp experiments (chain lengths 7–12). The numerical values of the two electrophysiologically determined affinities were similar (Fig. 5), and these in turn were similar to the values obtained in the binding experiments. Thus, it was not possible to conclude that the radioligand binding measurements preferentially reflect the interaction of the diamines with one or the other of the blocking sites. Most likely, inhibition of binding occurs via an interaction of the diamine with both the voltage-dependent and voltage-independent sites.

Discussion

Previous studies utilizing radioligand binding (4, 5) and electrophysiological recording (7, 8, 9) techniques have demonstrated that NMDA receptors are susceptible to block by long-chain *n*-alkyl diamines. For DA10 and DA12, a portion of the block occurs in a voltage-dependent manner and flickering of unitary NMDA receptor currents is observed in single-channel recordings. Taken together, these observations suggest an open channel blocking mechanism in which the positively charged diamine diffuses into and out of the cation permeation pathway, occluding ion transit during the periods of time that it is bound to an acceptor site in the pore. Despite the clear voltage-dependence of a fraction of the long-chain diamine block, we previously observed that there was a significant residual component of the block that could not be relieved at positive membrane potentials, raising the possibility that diamines can also inhibit NMDA receptor current as a result of their interaction with a distinct voltage-independent blocking site (7; see also Ref. 9).

In the present study, we further investigated the voltage-dependence of the block by diamines of various chain lengths in an attempt to verify the existence of this putative voltage-independent blocking site. The voltage-dependence curves for the long-chain diamines (carbon chain length > 6) exhibited a distinct plateau at intermediate levels of block, clearly demonstrating a voltage-independent component to the block. Thus, the blocking action of the diamines occurs at two distinct sites,

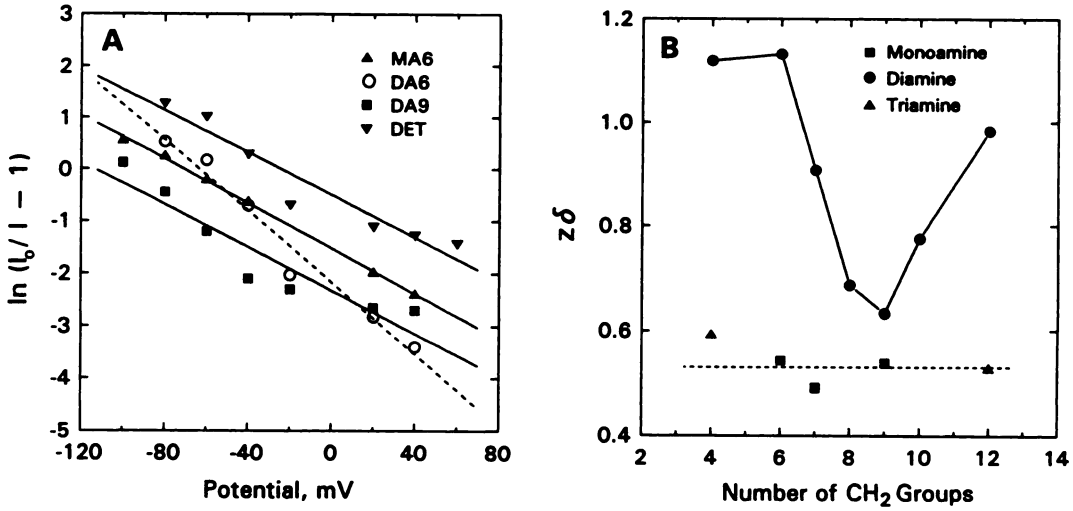

Fig. 6. *A*, voltage-dependence plots for a short (DA6) and intermediate (DA9) length diamine and a representative monoamine (MA6) and triamine (DET). The points indicate the means of data from 4–6 cells; the lines indicate the fits according to the equation given in the caption of Fig. 5. B, $z\delta$ values derived from plots similar to those illustrated in A for diamines of various carbon chain lengths and the monoamines MA6, MA7, and MA9 and the triamines DET and BHT. A concentration that produced approximately 50% block at −60 mV was used except for the longer diamines (DA7-DA12) and BHT, for which lower concentrations were used (~one-third the IC_{50} at −60 mV) to minimize block at the voltage-independent site.

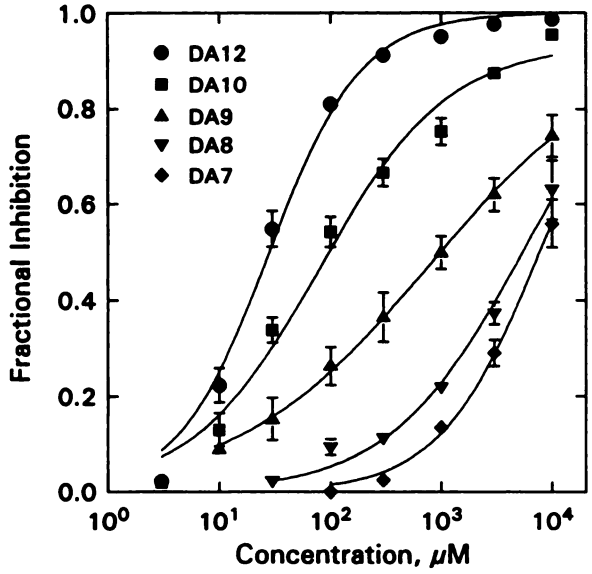

Fig. 7. Concentration-dependent inhibition of [^{3}H]dizocilpine binding to rat forebrain membranes by DA7, DA8, DA9, DA10, and DA12. The incubation was carried out in the presence of 100 μM glutamate and 100 μM glycine, as described in Methods. Each point is the mean ± SE of data from 3 separate experiments. The curves show the best fits to the data according to Eq. 1. The IC_{50} values obtained from the fits are given in Table I.

one which senses the membrane electric field and the other which does not. However, there are interesting similarities between the two sites. At both sites, two positive charges are necessary and sufficient for optimal affinity (*i.e.*, monovalent compounds show weak activity, whereas trivalent compounds have roughly equal or lower affinity than the corresponding diamine).

An additional similarity between the voltage-dependent and voltage-independent blocking sites is that the affinities of intermediate and long-chain diamines for the two sites (corrected for voltage) are numerically similar (Fig. 5). At both sites, binding affinity of intermediate and long-chain diamines increases with increasing chain length (equivalent to roughly a 600–700 cal/mol increase in binding energy per methylene group), suggesting that a hydrophobic interaction stabilizes binding of the diamines at each of the sites (18). In contrast, the affinity of short-chain compounds for the voltage-dependent site was not correlated with chain length, indicating that for the short diamines hydrophobicity is not an important factor in binding to this site. By analogy with divalent cation open channel blockers such as Mg^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+} (21, 22), the cationic nature of these short-chain compounds is likely to be of primary importance in determining binding. Divalent cations such as Zn^{2+} and Cd^{2+} can additionally inhibit NMDA receptors via an external, largely voltage-independent blocking site (23, 24), and it is attractive to consider whether the voltage-independent actions of the diamines could be mediated at this site. However, because short-chain diamines exhibited negligible voltage-independent block, this possibility seems unlikely, and we must tentatively conclude that the hydrophobic site where intermediate and long-chain diamines exert their voltage-independent block is distinct from the voltage-independent divalent cation inhibitory site.

Consideration of the voltage-dependence of the block produced at the voltage-dependent site provides insight into the dimension of the permeation pore of the NMDA receptor. As discussed above, results from whole-cell and single-channel recording strongly suggest that the voltage-dependent block produced by the diamines occurs by an open channel blocking mechanism so that it is reasonable to use the diamines as probes of the channel pore. The voltage-dependence of monoamines (Fig. 6B) and other monovalent cationic blockers (25, 26) indicate that NMDA receptors possess an amine binding site that is located 50–60% into the membrane field (measured from the outside). By analogy with the analysis of Miller (19) for block of K^+ channels by quaternary ammonium compounds, $z\delta$ values of the diamines can be used to provide a physical measure of the distance over which the membrane potential drop across the channel occurs. We assume that short- and intermediate chain diamines exert their block in the extended

configuration, their lowest energy conformation. We also assume that one of the charged groups binds to the same deep blocking site as monovalent channel blockers (whose mean δ value is 0.54). Divalent compounds blocking at this site would be expected to have twice the $z\delta$ value, and as noted above, this was the case for short diamines (DA4, DA6). Presumably, the two charged groups of these blockers can be accommodated at the deep blocking site. However, intermediate length diamines exhibited lower $z\delta$ values than the short diamines, and there was a progressive decrease in $z\delta$ value with increasing chain length until a minimum was reached with DA9. Miller (19) proposed that such a decrease could be due to the increasing physical separation of the second charged group from the charged group bound to the deep blocking site. As the second charge becomes closer to the edge of the electric field, it contributes less to the total $z\delta$ value, which represents the sum of the contributions of each charged group. Because the $z\delta$ value of DA9 is close to that of the monovalent blockers, its length provides an estimate of the depth of the deep blocking site. The intercharge distance of DA9 in the extended conformation, as estimated using the Polak-Ribiere molecular mechanics optimization algorithm (HyperChem), is 12.5 Å. If we make the further assumption that the electric field profile of the internal side of the pore is similar to that of the external side, the total distance over which the applied electric field drops would then be ~23 Å. As in the case of the sarcoplasmic reticulum K^+ channel (19), this distance is small compared to the thickness of the neuronal membrane (60–90 Å), suggesting that the NMDA receptor channel has large vestibules on one or both sides of a constriction over which the voltage drop occurs. Although our assumption of equivalence between the electric field profiles of the external and internal sides of the pore is hypothetical, the data of Johnson and Ascher (27) on the depth of the blocking site for intracellular Mg^{2+} is consistent with the simple model we have adopted and provides a basis for estimating the distance between the external and internal cation blocking sites. Thus, the sum of the electrical depths of the external deep blocking site ($\delta = 0.54$ in the present study) and the internal Mg^{2+} binding site ($\delta = 0.35$, measured from the inside; ref. 27) is slightly less than 1, providing room for a constriction between the external and internal blocking sites of electrical distance ~0.1. Assuming the electric field in this region of the channel is homogeneous with the diamine accessible region, this distance represents a physical separation of perhaps 2.5 Å (estimated as discussed above), a reasonable dimension of the permeation site/selectivity filter for an ion-selective channel (28). The complexity of the effects of external Mg^{2+} has precluded a comparable analysis using estimates of the electrical depth of the external Mg^{2+} blocking site (22). In this context, it will be of interest to determine if internal diamines and other organic cations produce a comparable voltage-dependent block as does internal Mg^{2+}.

We next consider the rather surprising observation that there is a reversal in the trend toward lower $z\delta$ values for the long-chain diamines DA10 and DA12. These compounds exhibited progressively larger $z\delta$ values that approached the $z\delta$ values of short-chain diamines and twice the $z\delta$ values of monovalent blockers. Miller (19) observed a roughly analogous situation in his studies of quaternary ammonium block of K^+ channels and proposed that the long-chain compounds have sufficient flexibility to allow them to bind in a bent-over conformation with both positively charged groups gaining access to the deep blocking site. A similar explanation seems applicable here. Recognizing that $z\delta$ represents an average, we presume that the progressive increase in the $z\delta$ value in going from DA10 to DA12 reflects the increased flexibility of DA12 and the correspondingly increased probability of it assuming the folded-over conformation. It is interesting that the 12-carbon triamine BHT has a $z\delta$ value near that of the monovalent blockers (and also markedly reduced affinity). Presumably, the centrally positioned NH_2 group restricts bending so that BHT behaves more like a monovalent compound, although it is possible that the additional charge interferes with binding to the deep blocking site. Indeed, the four-carbon triamine DET had a low $z\delta$ value, suggesting that it is not accommodated at the deep site.

It has been proposed that certain polyamines produce a voltage-dependent reduction of NMDA receptor current by screening fixed negative charges near to the opening of the channel pore (29, 30). We cannot exclude the possibility that charge screening to some extent contributes to the voltage-dependent inhibitory action of the diamines, particularly in the case of the short-chain compounds, which were only active at high concentrations. Indeed, preliminary single-channel recordings have indicated that short-chain diamines produce an apparent reduction in NMDA receptor single-channel conductance,[1] unlike DA10 and DA12, which, as noted above, induce flickering and are therefore likely to be open channel blockers (7). Such a reduction in single-channel conductance could be produced either by charge screening or by open channel block (if blocking and unblocking occur too rapidly to be resolved). However, several of our observations indicate that charge screening is not a major factor in the observed voltage-dependent blocking action of the diamines. First, the inhibitory effect exhibited by polyamines acting via charge screening is characterized by a shallow electrical depth ($\delta = 0.06$–0.13; Ref. 30), whereas we have shown that the diamine $z\delta$ values vary with chain length, and we argue that their voltage-dependent block occurs at a deep site. Second, if charge screening accounted for the inhibitory actions of the diamines, we would not expect there to be substantial affinity differences among diamines of different chain lengths. Third, trivalent compounds, by virtue of their increased charge, should have enhanced potency, whereas, as noted above, we observed that such analogs had equal or lower potencies than the corresponding diamines. Fourth, the concentration-response data (Fig. 2) were adequately fit by a model predicting complete block at sufficiently high diamine concentrations, whereas charge screening would not be expected to produce complete block of permeation. Thus, charge screening is unlikely to account for the blocking action of the diamines, even in the case of short-chain compounds.

As noted in the introduction, radioligand binding studies have indicated that DA10 may be an inverse agonist at the facilitatory polyamine site. Because this site is not located within the transmembrane field (9), the inverse agonist activity is presumably not exerted at a locus related to the voltage-dependent blocking site (see also Ref. 29). It is conceivable, however, that the voltage-independent component of the inhibition seen with the longer diamines is mediated at the same site where spermine produces its facilitatory action. In this

[1] S. Subramaniam, S.D. Donevan and M.A. Rogawski, unpublished observations.

case, the putative polyamine site antagonist DET should reverse the inhibitory action of DA10, as it does in binding studies (4). However, DET was inactive as an antagonist of DA10's blocking action at the voltage-independent site. Assuming DET is a true facilitatory polyamine site antagonist (a contention that has been disputed; see Refs. 9 and 30), DA10 does not appear to be an inverse agonist at the polyamine facilitatory site. We thus conclude that the diamine block and polyamine facilitation occur at distinct sites, and this is supported by binding studies which failed to observe a competitive interaction between various diamines and the facilitatory agonist spermidine (31).

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